UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION AND OPTIMIZATION OF CELLULASE PRODUCTION BY Aspergillus terreus USING OIL PALM EMPTY FRUIT BUNCH FIBRE IN SUBMERGED CULTURE

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By

MAHDI SHAHRIARINOUR

Thesis Submitted to the School of Graduate Studies, University Putra Malaysia, in Fulfilment of the Requirements for the Doctor of Philosophy

May 2011

DEDICATION

To my wife and my family

Abstract of thesis presented to the Senate of University Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

CHARACTERIZATION AND OPTIMIZATION OF CELLULASE PRODUCTION BY *Aspergillus terreus* USING OIL PALM EMPTY FRUIT BUNCH FIBRE IN SUBMERGED CULTURE

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MAHDI SHAHRIARINOUR

May 2011

Chairman : Associate Professor Mohd Noor B Abd Wahab, PhD

Faculty : Biotechnology and Biomolecular Science

The production of three major components of cellulase (FPase, CMCase and β-glucosidase) by *Aspergillus terreus* was studied in a shake flask experiment. The effects of physical, chemical and biological treatments on the oil palm empty fruit bunch (OPEFB) fibre for subsequent use as substrate for cellulase production were investigated. On the other hand, the effect on the surface morphology of the fibres, were examined under Scanning Electron Microscopy. The effects of different types and concentrations of nitrogen sources on cellulase production were also examined. The optimized medium composition obtained from the shake flask experiment was used for cellulase production in a 2L stirred tank fermenter where the effect of different levels of dissolved oxygen tension (DOT) at a fixed agitation speed on cellulase production was investigated. The experimental data obtained from batch fermentations in a shake flask

and the fermenter using the optimized medium were analysed to form the basis for a kinetic model of the process. The effect of different methods of treatment of OPEFB fibre on the rate and degree of hydrolysis was investigated. The use grinded OPEFB fibre increased cellulase production about two fold compared to 10-mm fibre. Chemical treatment with 0.5 % phosphoric acid significantly increased the cellulose and reduced the lignin contents. Cellulase activities, obtained from fermentation using OPEFB fibre treated with phosphoric acid followed by autoclaving at 160°C for 10 min and then treated biologically using effective microorganisms were about three times higher than those obtained in fermentation using untreated OPEFB fibre. The cellulase of *A. terreus* contained a high proportion of β-glucosidase with the ratio of specific activity of β-glucosidase to FPase of about 8. Yeast extract gave the highest cellulase production followed by peptone, urea and $(NH_4)_2S0_4$. A good agreement between the calculated data and the experimental data for both cell growth and cellulase production were observed, suggesting that the proposed model based on logistic and Luedeking-Piret equations is sufficient to describe the growth of *A. terreus* and cellulase production. The maximum activities of FPase, CMCase and β-glucosidase obtained from fermentation with 55 % DOT were 2.33, 51.1 and 16.18 U/mL, respectively. Cellulase production in stirred tank fermenter were significantly higher than that obtained in shake flask. The characterization of the proteins purified was shown that eight of the components were known; four endoglucanases (Endo I, II, III and IV), three exoglucanases (Exo I, II and III) and β-glucosidase were isolated from a cellulase preparation derived from *A. terreus*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah.

PENCIRIAN DAN OPTIMISASI PENGHASILAN SELULASE DARI *Aspergillus terreus* MENGGUNAKAN SUBSTRAT SERAT TANDAN KOSONG BUAH KELAPA SAWIT DALAM KULTUR TENGGELAM

Oleh

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Penghasilan tiga komponen utama selulase (selobiohidrolase, endoglukanase dan B-glukosidase) oleh kulat *Aspergillus terreus* telah dikaji dalam percubaan kelalang bergoncang. Kesan rawatan fizikal, kimia dan biologi terhadap penyediaan serabut tandan kelapa sawit kosong (TKSK) untuk digunakan sebagai substrat bagi penghasilan selulase telah dikaji. Di sudut lain, kajian telah dibuat dengan melihat kesan permukaan morfologi serat menggunakan Skan Elektron Mikroskop. Kesan dari bermacam jenis dan kepekatan sumber nitrogen terhadap penghasilan selulase juga telah dikaji . Komposisi medium optimum untuk penghasilan se1ulase yang diperoleh dari percubaan kelalang bergoncang telah digunakan untuk penghasilan selulase dalam tangki fermenter bergoncang berukuran 2 L dimana, turut dikaji kesan dari bermacam paras tegangan oksigen terlarut (DOT) pada kelajuan pengacau yang tetap, terhadap

penghasilan selulase. Data kajian yang diperoleh dari fermentasi sesekumpul dalam kelalang bergoncang dan fermenter yang menggunakan medium optimum telah dianalisa bagi membentuk model kinetik asas untuk proses tersebut . Kesan dari berbagai kaedah rawatan pada serabut TKSK terhadap kadar dan derajah hidrolisis telah dikaji. Penggunaan serabut TKSK yang dihaluskan telah meningkatkan penghasilan selulase sebanyak dua kali ganda berbanding dengan serabut TKSK 10-mm. Rawatan kimia menggunakan 0.5 % asid fosforik telah nyata meningkatkan kandungan selulosa dan menurunkan kandungan lignin . Penghasilan aktiviti selulase dari fermentasi menggunakan serabut TKSK yang dirawat dengan 0.5% asid fosforik dan diikuti dengan pengsterilan autoklaf pada suhu 160 °C selama 10 minit dan ini diteruskan lagi dengan rawatan biologi menggunakan mikroorganisma efektif dimana hasilnya adalah hampir tiga kali ganda lebih tinggi penghasilan selulase dari fermentasi menggunakan serabut TKSK tidak dirawat.. Selulase dari kulat *A. terreus* menggandungi paras β- glukosidase yang tinggi dengan nisbah aktiviti spesifik β-glukosidase terhadap FPase sebanyak 8. Ekstrak ragi telah memberikan penghasilan selulase yang tertinggi diikuti dengan pepton, urea, dan (NH4)2S04' . Satu keputusan yang hampir sama telah didapati antara nilai data yang dikira dengan data percubaan bagi pertumbuhan sel dan penghasilan selulase , ini menunjukkan bahawa model yang dicadangkan berasaskan persamaan logistic dan Luedeking-Piret adalah mencukupi bagi menerangkan pertumbuhan sel *A. terreus* dan penghasilan selulase. Aktiviti maksimum yang diperolehi bagi FPase,CMCase dan B-glukosidase dari fermantasi dengan 55% DOT adalah masing-masing, 2.33, 51.1 dan 16.18 U/ml. Penghasilan selulase dari fermenter tangki bergoncang adalah nyata lebih tinggi jika dibandingkan dengan penghasilan selulase dari kelalang bergoncang. Hasil dari pencirinan protein tulen didapati lapan komponen telah dikenal pasti iaitu; empat endoglukanase (Endo I, II, III dan IV), tiga eksoglukanase (Exo I, II dan III) dan β-glukosidase telah dipisahkan dari persediaan selulase *A.terreus* .

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I would like to thank and be grateful to my wife, for helping me throughout my studies, so I could complete my work in success.

I certify that an Examination Committee met on 27th May 2011 to conduct the final examination of Mahdi Shahriarinour on his Doctor of Philosophy thesis entitled "Characterization and Optimization of Cellulase Production by *Aspergillus terreus* Using Oil Palm Empty Fruit Bunch Fibre in Submerged Culture" in accordance with Universities and University Colleges Act 1971 and the constitution of the university Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy. Members of the Thesis Examination Committee were as follows:

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I declare that the thesis is my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institutions.

MAHDI SHAHRIARINOUR

Date: 27 May 2011

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Four major factors that lead to the renewed interest in lignocellulose research are (i) the increase in demand from the third world countries of modern materials, (ii) changes in the economics of competing materials, (iii) a renewed concern for our environment, and (iv) global interest in recycling. After a thousand years, lignocellulose is once again emerging as a resource of precursors for the production of polymeric materials, organic chemicals and fuel. The bioconversion process for converting lignocellulose into fermentable sugars involves three steps, namely, (i) pretreatment of the substrate, (ii) production of the cellulase enzymes and (iii) hydrolysis of the pretreated substrate. Much effort in cellulase research has been directed toward cellulolytic microorganisms, as well as to the structure, function and synergistic activity of these enzymes. Cellulase is a complex or a mixture of enzymes that act in concert to hydrolyze crystalline cellulose to glucose. It is known that cellulase consists of three classes of enzymes, cellobiohydrolases (CBH, EC 3.2.1.91), cleave cellobiosyl units in the ends of cellulose chains, endoglucanases (EG, EC 3.2.1.4), cleaving middle glucosidic bonds and β-glucosidase (EC 3.2.1.21), cleaving glucose units from cello-oligosaccharides. Reaction conditions and the production rate of the related enzyme system extensively influence the production of the enzyme-based bioconversion technology.

Pretreatment usually targets hemicellulose, lignin or both, in order to make biomass more accessible. For many years, researchers have been trying to find the alternative energy sources for the transport fuel because fossil energy is limited. Lately, the focus has been changed to renewable bioenergy. To achieve low-cost bioethanol, using lignocellulosic materials is one of the options. Lignocellulosic materials contain abundant polysaccharides. Pretreatment can help polysaccharides and sugars to be liberated. The cellulooligomers, known as polysaccharides with enormous molecular weight, not only require cellulase to perform the hydrolysis of polysaccharides but also induce cellulases. Therefore, the low-cost bioethanol is achievable with an economic cellulase production process by optimizing the factors of culture condition and using lignocellulosic hydrolysate.

In Malaysia, the overall performance of the sector is largely attributed to a large increase in the production of palm oil. Palm oil has emerged as the fastest growing commodity oil in the global market, helping to meet the demands from both the food and non-food sectors. The oil palm tree itself is the most efficient oil bearing crop with an ability to produce oil yield almost 6-10 times higher than the other oil-bearing crops. The palm oil industry also contributes to the rising need for biodiesel. Indeed, there could be a major oil shortage without Malaysian palm oil to cater for the current food and fuel demands. Cellulase refers to a division of enzymes produced mostly by fungi, bacteria, and protozoans that catalyse the cellulolysis (or hydrolysis) of cellulose. On the other hand, there are also cellulases produced by other type of organisms such as plants and animals. Several different kinds of cellulases are known, which vary structurally and mechanistically (Chapin et al., 2006). Scientific community have tough interests in cellulases because of their applications in industries as follows: starch processing, animal food production, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry, and textile industry (Adsul et al., 2007; Kaur et al., 2007). However, the major bottleneck of inclusive application of cellulase in industry is the high cost of the enzyme production. Great cost decrease may be possible by exploring ways of cellulose change by using microorganisms that produce cellulolytic enzymes. It is so essential to look for microorganisms that have a high rate of cellulase production (Kotchoni and Shonukan, 2002).

The commercial cellulases are mostly extracellular enzymes produced by mesophilic or thermophilic fungi (Kim et al., 2005). Since the use of cellulose, degrading enzymes are related to industrial processing and operating at high temperature, application of thermostable enzymes produced by mesophilic or thermophilic fungi appears to be advantageous. *Aspergillus* species are major agents of decompositions, decay, and thus possess the capability to produce a wide range of enzymes. Cellulase production has been described for various *Aspergillus* species (Lockington et al., 2002; Ong et al., 2004; Wang et al., 2006), but only a few reports available on produce cellulase production from *Aspergillus terreus* (Emtiazi et al., 2001; Workman and Day, 1982). So far, few studies have done on the cellulase production of *A. terreus*. The rate of cellulase production is influenced by many factors, such as, environmental conditions, medium composition and hydrodynamic conditions within the fermenter. The effect of different types of nitrogen sources on cellulase production have been investigated (Chaabouni et al., 1995; Gottvaldova et al., 1982; Tong and Rajendra, 1992; Umikalsom et al., 1997b). However, the effects of the concentrations of nitrogen sources on the ratio activities of three major components of the cellulase complex have not been well quantified. Although the effect of agitation intensity or shear rate on the cellulase producing activity of fungus has been investigated (Mukataka et al., 1988; Wase et al., 1985), very little attention has been paid to the influence of dissolved oxygen tension in the culture during fermentation at a fixed agitation speed on the synthesis of all the three major components of cellulase. Since very little report on the effect of dissolved oxygen level on cellulase production is available in literature, study on this aspect deserves further detailed investigated.

1.2 SIGNIFICANT OF STUDY

The major aim of cellulase application is for the conversion of lignocellulosic materials into fermentable sugars. During production of bioethanol, the cost of the enzymes used for hydrolysis needs to be reduced and the enzyme's efficiency needs to be enhanced to make the economically feasible. The enzyme production costs are firmly connected with the productivity of enzyme-producing microbial strain and the final (protein) yield in the fermentation. Producing cellulase is a key factor in the hydrolysis of cellulosic material and it is important to make the process economically viable. Therefore, the scope of research here falls into the examination of the cellulase production using various treatment methods and the examination of the factors affecting cellulase production.

In the industrial processing of oil palm fruit into oil, a large amount of oil palm empty fruit bunch (OPEFB) is generated. Having little commercial value, they become a disposal problem due to bulk density, thus occupying large storage volume and usually employed as fuel in the factory. As a lignocellulosic material, OPEFB represent a renewable and low cost material resource for production of fermentable sugars or for transformation into chemicals and other high value-added products. OPEFB is available in large quantities and has fairly high cellulose content with an average of 50% based on an oven dried basis; it appears to be potential substrate for cellulase production. Several studies in the literature indicated that the carbon source used in cultivations is one of most important factors affecting the cost and yield of cellulase production. Therefore, an economic and viable pretreatment process for lignocellulosic materials should be investigated.

1.3 RESEARCH OBJECTIVES

The specific objectives of the present project were;

(1) To screen and isolate the cellulase producer of fungi from local sources that have high ability to degrade OPEFB fiber.

(2) To investigate the effect of different chemical, physical and biological treatments on OPEFB fibers for subsequence usage as substrate in cellulase production by the isolated fungi.

(3) To optimize medium composition and growth factors for the production of cellulase enzyme by the isolated strain in the shake flask technique.

(4) To optimize the environmental conditions for cellulase production and kinetics of cellulase fermentation by the isolated strain, used OPEFB as substrate in the 2 L stirred tank fermenter.

(5) Purification and characterization of cellulase enzyme.

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